Mutants in *Arabidopsis thaliana* Altered in Epicuticular Wax and Leaf Morphology[1]

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We report eight new mutants in *Arabidopsis thaliana* possessing altered leaf morphology and epicuticular wax. These were isolated from a T-DNA-mutagenized population using a visual screen for altered leaf reflectance, i.e. increased glaucousness or glossiness. The mutants were placed into three distinct classes based on alterations in overall plant morphology: *knobhead* (*knb*), *bicentifolia* (*bcf*), and *wax*. The four *knb* mutants formed callus-like growths in the axillary region of the rosette leaves and apical meristem, the two *bcf* mutants produced hundreds of narrow leaves, and the two *wax* mutants had leaves and stems that were more glossy than wild type and organs that fused during early development. Leaves of *knb* and *bcf* were more glaucous and abnormally shaped than wild type. Epicuticular wax crystals over *knb* and *bcf* leaf surfaces (where none were present on wild type) likely contributed to their more glaucous appearance. In contrast, the glossy appearance of the *wax* mutants was associated with a reduced epicuticular wax load on both leaves and stems. One representative from each phenotypic class was selected for detailed analyses of epicuticular wax chemistry. All three lines, *knb1*, *bcf1*, and *wax1*, had dramatic alterations in the total amounts and relative proportions of their leaf epicuticular wax constituents.

Epicuticular waxes form the outermost layer of aerial plant organs and are thought to confer resistance to insect herbivory, fungal pathogens, and drought (Jordan et al., 1984; Jenks et al., 1994a; Eigenbrode and Espelie, 1995). The production of plant epicuticular wax is a biologically complex process involving a host of synthetic and transport mechanisms (Kolattukudy, 1975; Hallam, 1980; Jenks et al., 1994b, 1995; Wettstein-Knowles, 1995; M.A. Jenks, K.A. Feldmann, unpublished data). Through a compilation of previous mutation analyses (Wettstein-Knowles, 1982; Bianchi et al., 1985; Hannoufa et al., 1993) and labeling studies (Kolattukudy, 1975; Wettstein-Knowles, 1982), a rudimentary model has been set forth to describe potential enzymatic and substrate transfer steps in the Arabidopsis leaf epicuticular wax biosynthetic pathway (Jenks et al., 1995; M.A. Jenks, K.A. Feldmann, unpublished data). However, mechanisms for secretion of epicuticular wax precur-

sors by leaf epidermal cells have been given little attention (Anton et al., 1994; Jenks et al., 1994b).

Most epicuticular wax is secreted to the plant surface after cell-wall and cuticle layers are well developed (Jenks et al., 1994b). Since microscopic channels for epicuticular wax secretion through plant epidermal cell walls and cuticles are not visible at the level of the transmission electron microscope (Jeffree et al., 1976; Anton et al., 1994; Jenks et al., 1994a, 1994b), epicuticular wax precursors may travel to the plant surface by diffusion in submicroscopic spaces (Jeffree et al., 1976). Theoretically, a single gene mutation that alters physical properties or organization of microspaces in the cell wall or cuticle could change the epicuticular wax phenotype by altering epicuticular wax secretory pathways. Moreover, alterations in cytoplasmic secretory pathways (Barinaga, 1993) could alter the deposition of epidermal cell secretory products like cell-wall and cuticle constituents.

Leaf epicuticular wax mutants were selected by visual changes in leaf surface glaucousness of *Sorghum bicolor* (Jenks et al., 1992), *Zea mays* (Lorenzoni and Salamini, 1975), *Brassica oleracea* (Anstey and Moore, 1954; Macey and Barber, 1970a), and *Pisum sativum* (Macey and Barber, 1970b). Epicuticular wax mutants in *Arabidopsis thaliana* designated *cer* were previously selected from a mutagenized Arabidopsis population based on visibly decreased glaucousness of the stem surface (Koornneef et al., 1989; McNevin et al., 1993). The visual surface glaucousness and electron-microscopic appearance of Arabidopsis wild-type and *cer* leaves were identical even though the chemical composition of epicuticular wax on most *cer* leaves was different from that of the wild type (Jenks et al., 1995). To date, leaf epicuticular wax mutants in Arabidopsis with visibly altered leaf glaucousness have not been reported. Leaf epicuticular waxes play a critical role in plant environmental stress resistance and their genetic modification therefore has great potential for crop improvement. However, genetic control of epicuticular wax biosynthetic and secretory processes must be understood before serious programs to engineer crop epicuticular waxes are begun.

We initiated a study to screen for Arabidopsis leaf epicuticular wax mutants in a T-DNA-mutagenized population based solely on leaf surface glaucousness. We isolated eight mutants with altered leaf reflectance and placed them

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Abbreviations: *bcf*, *bicentifolia*; *cer*, *eceriferum*; *fdh*, *fiddlehead*; *knb*, *knobhead*; WS, Wassilewskija.

into three distinct classes based on overall plant morphology. These were designated knb, bcf, or wax. All eight lines had altered leaf and stem epicuticular wax crystallization patterns. One representative from each phenotypic class was selected for detailed chemical analyses of its epicuticular wax constituents, and those results are presented.

MATERIALS AND METHODS

Plant Material

Arabidopsis thaliana, ecotype WS, was previously transformed with Agrobacterium tumefaciens to generate mutant transgenic families (Feldmann and Marks, 1987; Feldmann, 1991; Forsthoefel et al., 1992). We screened a collection of 14,000 T-DNA-generated Arabidopsis transformants for mutants having visual alterations in leaf surface glaucousness. Six mutants with increased leaf glaucousness and two mutants with increased leaf glossiness (decreased glaucousness) were selected, and these were divided into three classes based on leaf morphology. Plants were grown in a controlled environmental chamber (Conviron, Winnipeg, Manitoba, Canada) at 22°C and for 16-h d (approximately 240 μmol m^{-2} s^{-1}; 75 to 95% RH; 20°C dark temperature).

Genetic Analyses

Because three of the knb mutants and the two wax mutants were sterile, these mutants were maintained as heterozygotes. To test dominance relationships, progeny from wild-type plants of each line were scored for mutant and wild-type phenotypes. To test for co-segregation of the visible defects in each line, approximately 1000 progeny from plants known to segregate for these phenotypes were planted in flats and grown to maturity. Plants not displaying any of the recessive phenotypes were culled from the population. The remaining plants in each population were scored for the respective mutant phenotypes.

Micro-Structural Analyses

The leaf and stem tissues from the selected mutants were air-dried before sputter coating with gold-palladium using short, 30-s repeated bursts to prevent melting of epicuticular wax crystals. All samples were collected from 25-d-old plants except bcf1 stems, which were collected from 38-d-old plants. Leaf and stem tissues were also chemically preserved in formalin-alcohol-acetic acid, dehydrated in a graded ethanol series, and critical-point dried before sputter coating. Specimens were examined using a scanning electron microscope at 10 kV. Electron micrographs of leaf and stem surfaces from three or more replicate plants were examined for each line. Average leaf areas were determined from 75 leaves by computer digitization.

Chemical Analysis

The hexane-soluble surface lipids (designated epicuticular wax) were extracted from 25-d-old plants by immersing separate leaf and stem tissues in hexane for 30 s. Derivatization, GC, MS, and quantification of identified epicuticu-

lar wax constituents were performed according to Jenks et al. (1995).

Chemicals identified as epicuticular wax constituents on Arabidopsis were tetradecanoic, hexadecanoic, octadecanoic, eicosanoic, docosanoic, tetracosanoic, hexacosanoic, octacosanoic, and triacontanoic acids as C_{14}, C_{16}, C_{18}, C_{20}, C_{22}, C_{24}, C_{26}, C_{28}, and C_{30} free fatty acids; n-pentacosane, n-heptacosane, n-nonacosane, n-hentriacosane, and n-tritriacontane as C_{25}, C_{27}, C_{29}, C_{31}, and C_{33} alkanes; 1-tetracosanol, 1-hexacosanol, 1-octacosanol, 1-triacontanol, and 1-dotriacontanol as C_{24}, C_{26}, C_{28}, C_{30}, and C_{32} primary alcohols; tetracosanal, hexacosanal, octacosanal, and triacontanal as C_{24}, C_{26}, C_{28}, and C_{30} aldehydes; 13- and 14-heptacosanol, 14- and 15-nonacosanol, and 15- and 16-hentriacontanol as C_{27}, C_{29}, and C_{31} secondary alcohols; and 15-nonacosanone as C_{29} ketone.

RESULTS

Morphological Analysis

We visually screened 14,000 segregating families of Arabidopsis that were generated by T-DNA insertion mutagenesis and selected six mutants having increased leaf surface glaucousness (increased whitish cast) and two having increased leaf surface glossiness (increased shininess and/or spectral reflectance). The six glaucous mutants fell into two distinct morphological classes designated knb and bcf, whereas the two glossy lines represented a third morphological class designated wax (Fig. 1, A–D, and data not shown). A general analysis of all eight novel epicuticular wax mutants with more detailed morphological, developmental, and chemical analyses of one representative from each of the three morphological classes is presented herein.

knb Mutants

The knb mutants had leaves that were more glaucous and rumpled than leaves of wild type (Fig. 1, A and B, and data not shown). knb mutants also formed a dark-green, callus-like growth at axillary regions of rosette leaves, which in turn eventually gave rise to short, finger-like projections (not shown). One mutant, designated knb1, possessed larger leaves, whereas the other three knb mutants had much smaller leaves than wild type (Fig. 1, A and B, and data not shown). These small knb mutants were morphologically distinguishable from each other by leaf shape, size, and degree of callus proliferation (data not shown). As knb mutants aged, axillary buds of all four mutants and apical meristems of the three small mutants were engulfed by the callus (A. Traut, M. Jenks, and K. Feldmann, unpublished data). The knb1 mutant produced a primary inflorescence (stem) that was glaucous like wild type, whereas the three small knb mutants did not produce stems.

The knb1 mutant was selected for detailed characterization of its epicuticular wax constituents (presented below) and morphology because it was fertile and had large leaves. Individual leaf areas of 25-d-old knb1 plants were 199 mm^2 compared to wild type at 172 mm^2 (Table
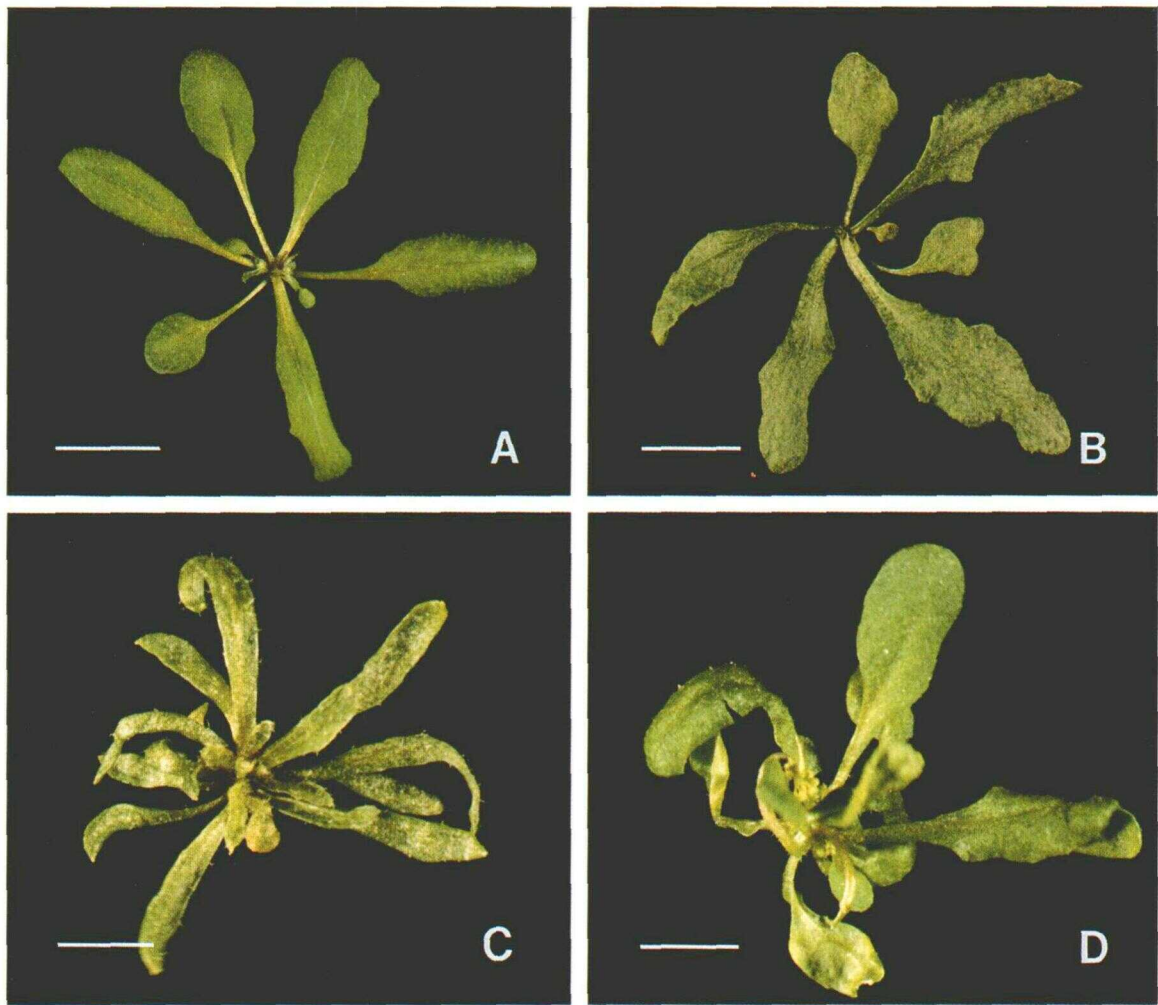
Figure 1. Rosettes with stems removed. A, Wild type, (bar = 1 cm); B, *knb1* (bar = 1 cm); C, *bcf1* (bar = 0.5 cm); D, *wax1* (bar = 0.5 cm).

I). Leaf epidermal cells of *knb1* (visualized using a scanning electron microscope and chemically preserved tissues) appeared larger, more irregularly shaped, less organized, and more puffed out above the leaf surface plane than wild-type leaf epidermal cells (data not shown).

bcf Mutants

The two mutants designated *bcf* had leaves that were more glaucous and narrow than wild type (Fig. 1, A and C, and data not shown). In addition, *bcf* mutants had many more leaves than wild type at 25 d and continued to proliferate leaves long after the wild-type plants began

senescence. Leaf counts made for both *bcf* mutants after 45 d of growth exceeded 100 total leaves (data not shown). *bcf* mutants initiated bolting roughly 30 d after germination compared to 15 d for wild-type plants under our growth conditions (data not shown). *bcf* mutants had greatly reduced fertility. These two *bcf* mutants were distinguishable from each other by leaf origin and number (data not shown).

The *bcf1* mutant was selected for detailed characterization of its epicuticular wax constituents (presented below) and morphology. At d 25, the average number of leaves per plant on *bcf1* was 18.5, whereas wild type produced 6.1 (Table I). The average individual leaf area per *bcf1* plant at 25 d was 31 mm^2 (Table I). Similar to

Table I. *Leaf number per plant and individual leaf area of 25-d-old Arabidopsis wild-type WS, knb1, bcf1, and wax1*

Parameter	WS	knb1	bcf1	wax1
Leaf No.[a]	6.2 ± 1.2	5.6 ± 1.3	18.5 ± 7.2	5.5 ± 2.0
Leaf area (mm^2)[b]	172 ± 13	199 ± 75	31 ± 20	45 ± 22

[a] Average number of rosette leaves on 10 plants (mean ± SD).   [b] Average individual adaxial plus abaxial leaf blade area of 75 leaves (mean ± SD).

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knb1, leaf epidermal cells of *bcf1* appeared larger and were more irregularly shaped, less organized, and more puffed out above the leaf surface plane than wild-type leaf epidermal cells (data not shown).

wax Mutants

The two *wax* mutants had leaves that were smaller and more glossy and irregularly shaped than wild type (Fig. 1, A and D, and data not shown). *wax* mutant stems were also more glossy and slender than wild type (data not shown). In addition, *wax* mutant stems had a twisting (or spiraling) appearance. It is interesting that *wax* mutant organs fused during early organ development (e.g. leaf to leaf, leaf to stem, sepal to sepal). Fusions were observed between epidermal cells of most aerial organs with connections at regularly distributed attachment points along the fusion borders that resembled "suture-like connections" (as described by Lolle et al., 1992; data not shown). Both *wax* mutants were male and female sterile and had an indeterminate growth habit (continuing to produce many new branches long after wild-type plants began to senesce). The two wax mutants were distinguishable from each other only in that *wax1* was larger than the second *wax* mutant.

The *wax1* mutant was selected for detailed characterization of its epicuticular wax constituents (presented below) and morphology. Although the average number of leaves on *wax1* was similar to that of wild type, the average individual leaf area of *wax1* was 45 mm^2 compared to wild type at 172 mm^2 (Table I). Epidermal cells of *wax1* were smaller and, except at organ-fusion sites, similar to wild type in shape (data not shown).

Microscopic Epicuticular Wax Crystallization Patterns

Wild-type Arabidopsis adaxial (Fig. 2A) and abaxial leaf surfaces (see Jenks et al., 1995) lacked epicuticular wax microscopic crystals. All *knb* and *bcf* mutants produced flake-like epicuticular wax crystals over both the abaxial and adaxial leaf surfaces (Fig. 2, C and E, and data not shown). The wax crystals on *knb1* mutants were larger than those on *bcf1* mutants (Fig. 2, C and E). Moreover, *knb* mutant wax crystals uniformly covered the entire leaf surface, whereas *bcf* wax crystals were more densely distributed near the leaf margins (data not shown). Like the wild type, *wax* mutants lacked epicuticular wax crystals on their leaves (Fig. 2, A and G).

Stem epicuticular wax crystals on wild type, *knb1*, and *bcf1* appeared to have only small differences in microscopic morphology (Fig. 2, B, D, and F). Compared to wild type, *knb1* had more smaller, plate-like wax crystals, and *bcf1* had more numerous and smaller, plate-like crystals plus many long, slender, ribbon-like wax crystals. In contrast, stems of *wax1* had fewer epicuticular wax microscopic crystals than wild type (Fig. 2, B and H). *wax1* mutant stems were particularly deficient in plate-like wax crystals (Fig. 2, B and H).

Genetic Analyses

To determine whether the multiple phenotypes of these novel mutants were due to a single or to multiple mutations, *knb1*, *bcf1*, and *wax1* mutants were examined for segregation of traits in a segregating population. A total of 255 mutant plants from a plant heterozyous for *knb1* were scored for the occurrence of rumpled leaves and increased leaf surface glaucousness; 205 mutant plants from a segregating *bcf1* family were scored for narrow leaves, increased leaf number, and increased leaf glaucousness; and 233 mutant plants from a segregating *wax1* family were scored for glossy leaves and stems, slender stems, rumpled leaves, and fertility. For each segregating population, the distinguishing traits for each mutant always co-segregated together. More than 600 wild-type plants were examined in each of these segregating populations and were found to lack any of the respective recessive traits. Therefore, the complex phenotypes for *knb1*, *bcf1*, and *wax1* are likely to be pleiotropic effects of a single nuclear recessive gene (data not shown).

Compositional Analysis of Epicuticular Wax

Leaf and stem epicuticular wax chemical constituents were analyzed on wild type, *knb1*, *bcf1*, and *wax1*. The total epicuticular wax load per leaf area was 1.6-fold higher on *knb1* than on wild type (Table II). The amount of free fatty acids, aldehydes, alkanes, and primary alcohols (1-alcohols) on *knb1* leaves were 1.8-, 1.4-, 1.6-, and 1.6-fold higher, respectively, than on wild-type leaves (Table II). The *knb1* leaf C_{29} alkanes were 1.5-fold higher, the C_{31} alkanes were 1.6-fold higher, and the C_{33} alkanes were 1.4-fold higher than those of wild type (Fig. 3). The *knb1* leaf C_{26} and C_{28} 1-alcohols were each 1.7-fold higher, whereas the C_{30} and C_{32} 1-alcohols were similar to wild type (Fig. 3). The largest difference in an epicuticular wax constituent class between wild-type and *knb1* leaves occurred in the ester fraction, which was 47-fold higher on *knb1* leaves (Table II).

The *knb1* stem total epicuticular wax load was similar to that of wild type (Table III). Aldehydes and alkanes were slightly lower, whereas 1-alcohols were higher on *knb1* stems than on wild-type stems (Table III).

The leaf epicuticular wax load on *bcf1* was similar to that of wild type (Table II). However, the amounts of free fatty acids and aldehydes on *bcf1* leaves were 2.3- and 1.2-fold higher, respectively, than on wild type (Table II). In contrast, the amounts of 1-alcohols on *bcf1* leaves were 3.5-fold lower than on wild-type leaves, whereas the alkanes on *bcf1* leaves changed little (Table II). The *bcf1* leaf C_{29} alkanes were 1.6-fold higher, the C_{31} alkanes changed little, and the C_{33} alkanes were 1.6-fold lower than wild type (Fig. 3). The *bcf1* leaf C_{26} 1-alcohols were 22.8-fold lower and the C_{28} 1-alcohols were 6.9-fold lower, whereas the C_{30} 1-alcohols changed little and the C_{32} 1-alcohols were roughly 2-fold lower than wild type (Fig. 3). The esters on *bcf1* were 18-fold higher than on wild type (Table II).

The *bcf1* stem total epicuticular wax load was similar to that of wild type (Table III). Aldehydes were slightly higher on *bcf1* stems (Table III).
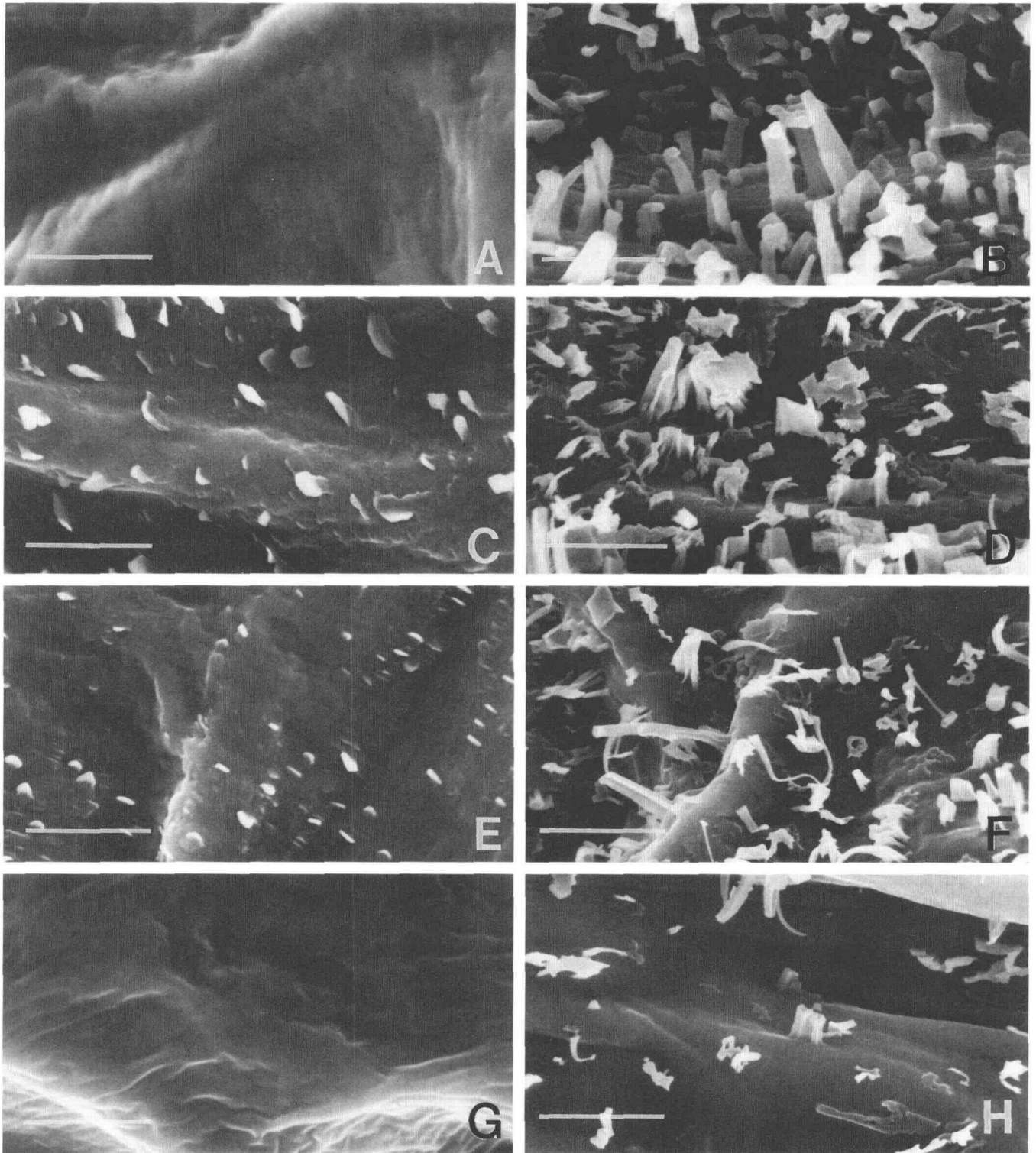
Figure 2. Microscopic surface morphology of leaves and stems using the scanning electron microscope. A, Adaxial leaf surface of wild type; B, stem surface of wild type; C, adaxial leaf surface of *knb1*; D, stem surface of *knb1*; E, adaxial leaf surface of *bcf1*; F, stem surface of *bcf1* (38 d); G, adaxial leaf surface of *wax1*; H, stem surface of *wax1*. Samples were air dried. Stem tissues were collected at mid-stem height. Bar = 5 μm.

Table II. *Leaf epicuticular waxes on 25-d-old Arabidopsis wild-type WS, knb1, bcf1, and wax1*

Values represent epicuticular wax load in μg dm^{-2} of total leaf surface areas.

Chemical Constituent	WS[a]	knb1	bcf1	wax1
Total wax load[b]	156	244	148	42.3
Free acids	4.0	7.2	9.2	5.1
Aldehydes	2.4	3.4	2.9	2.0
Alkanes	90.3	141	101	16.4
1-Alcohols	37.0	60.1	10.5	5.0
2-Alcohols	0.4	1.0	0.2	0
Ketones	1.2	1.3	0.7	0.3
Esters	0.2	9.4	3.7	0.1
Amyrins	6.5	6.9	6.4	5.1
Other terpenoids	7.6	8.6	10.4	4.9
Unknowns	6.7	5.4	3.3	3.5

[a] Wild-type values previously presented in Jenks et al. (1995). WS and mutant wax extractions were made concomitantly.   [b] SD values for total wax loads from three bulked samples of WS, knb1, bcf1, and wax1 leaves were 27.6, 60.3, 30.0, and 10.8, respectively. Values were calculated using an internal standard (hexadecane) and corrected peak areas from the gas chromatograph.

The total epicuticular wax load on *wax1* leaves was lower than on wild-type leaves by 3.7-fold (Table II). Whereas leaves of *wax1* had 1.3-fold more free fatty acids than wild-type leaves, leaf alkanes and 1-alcohols were 5.5- and 7-fold lower, respectively (Table II). The chain-length distribution for leaf alkanes and 1-alcohols on *wax1* was similar to that on wild type (Fig. 3). The amount of esters on *wax1* leaves changed little from wild-type leaves (Table II).

The total epicuticular wax load on *wax1* stems was lower than on wild-type stems by 2.5-fold (Table III). The stems of *wax1* had lower amounts of free fatty acids, aldehydes, alkanes, and 1-alcohols than did wild-type stems, with the greatest reduction occurring in the aldehyde and alkane portions (Table III). The *wax1* mutant stems had higher proportions of 2-alcohols and ketones to alkane than wild type and the other mutants presented herein (Table III). The ratio of C_{29} 2-alcohol/C_{29} alkane and C_{29} ketone/C_{29} alkane on wild-type stems was 0.3 and 0.6, respectively. Similar ratios were observed for *knb1* and *bcf1*. However, the C_{29} 2-alcohol/C_{29} alkane and C_{29} ketone/C_{29} alkane ratios in *wax1* stems were 3.9 and 8.0, respectively.

DISCUSSION

We report here the results of a visual screen for mutants among a T-DNA-induced population of Arabidopsis having altered leaf reflectance, i.e. increased glaucousness or glossiness. Evidence suggested that many more Arabidopsis genes whose products control epicuticular wax biosynthesis and/or secretion could be identified. For instance, the 21 Arabidopsis *CER* loci, previously described by Koornneef et al. (1989) and Hannoufa et al. (1993), were identified using a visual screen for reductions in stem glaucousness. Most of these *cer* mutants were also found to have altered leaf epicuticular waxes even though their leaves were visually identical to leaves of wild type (Jenks et al., 1995; M. Jenks, unpublished data). Only one allele

had been isolated for eight of these *CER* loci, suggesting that all of the genes involved in epicuticular wax biosynthesis and secretion had not been identified. Moreover, epicuticular wax constituents on *cer* leaves and stems were used to predict *CER* gene functions in models, illustrating the likely Arabidopsis epicuticular wax biosynthetic pathways (Lemieux et al., 1994; Jenks et al., 1995). Existing *cer* mutations could not be assigned to all of the necessary steps in these wax biosynthetic models, suggesting that gene mutations affecting these sites in the pathways had not yet been found. Furthermore, visual screens to identify leaf epicuticular wax mutants by altered visual leaf glaucousness had not been reported for Arabidopsis. Our objective was to identify novel epicuticular wax mutants
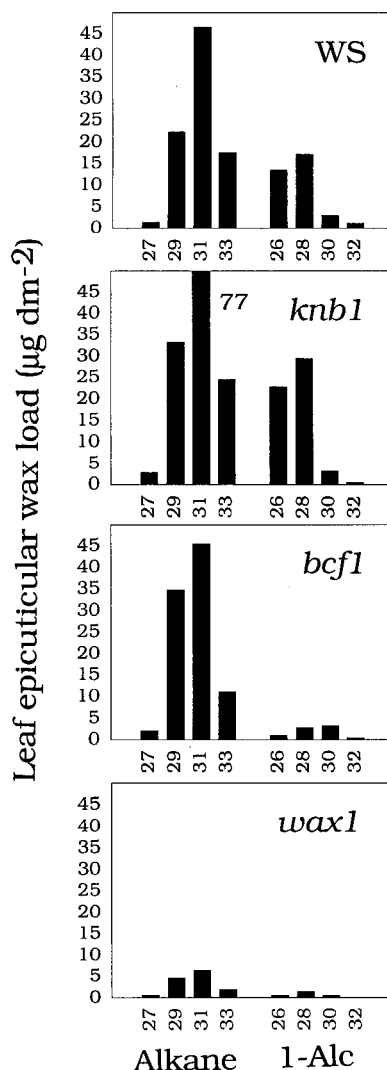

Figure 3. Leaf epicuticular wax chemistry of the Arabidopsis WS wild type and the leaf epicuticular wax mutants. Chemical classes and chain-length distributions are labeled on the horizontal axis for each. The total amount of each wax chemical class in μg dm^{-2} of surface area is labeled on the vertical axis. Where the wax constituent amount was off scale, a number designating the actual value is presented next to the bar. 1-Alc, 1-Alcohol.

Table III. *Stem epicuticular waxes on 25-d-old Arabidopsis wild-type WS, knb1, bcf1, and wax1*

Values represent epicuticular wax load in μg dm^{-2} of stem surface areas.

Chemical Constituent	WS[a]	knb1	bcf1[b]	wax1
Total wax load[c]	1620	1680	1560	642
Free acids	33.3	36.2	25.2	21.2
Aldehydes	45.0	31.2	86.7	3.7
Alkanes	721	653	687	39.1
1-Alcohols	104	158	95.7	80.0
2-Alcohols	181	200	168	123
Ketones	411	414	325	243
Esters	47.6	47.6	51.4	33.5
Amyrins	16.0	16.0	9.9	20.3
Other terpenoids	33.4	37.1	36.7	43.0
Unknowns	29.0	29.0	24.2	36.4

[a] Wild-type values previously presented in Jenks et al. (1995). WS and mutant wax extractions were made concomitantly.   [b] Epicuticular wax constituent loads determined from 38-d-old *bcf1* stems (average of two replicates).   [c] SD values for total wax loads from three bulked samples of WS, *knb1*, and *wax1* leaves were 16.9, 98.0, and 28.8, respectively. Values were calculated using an internal standard (hexadecane) and corrected peak areas from the gas chromatograph.

whose phenotypes would shed light on epicuticular wax biosynthetic and secretory processes.

Our screen of a T-DNA-mutagenized population identified eight mutants with alterations in leaf surface glaucousness. These mutants were divided into three classes based on overall plant morphology. Four mutants designated *knb* had more glaucous and rumpled leaves than wild type and had callus formations at axillary regions of the rosette leaves. Callus of *knb* mutants engulfed axillary buds and apical meristematic regions as the plants aged. Two mutants designated *bcf* had greater leaf numbers and more glaucous and narrow leaves than wild type. Two other mutants designated *wax* had glossier leaves and stems than wild type, irregularly shaped leaves and stems, and an organ-fusion phenotype.

The *knb* and *bcf* mutants produced flake-like epicuticular wax microscopic crystals over the adaxial and abaxial leaf surfaces but none were present on the wild type. These wax crystals likely contributed, in part, to the glaucous appearance of *knb* and *bcf* leaves. To our knowledge, mutants having epicuticular wax crystals on leaves have not been reported in plant species that normally lack leaf epicuticular wax crystals. Moreover, *knb1* is the first reported plant mutant with increased total epicuticular wax load. However, *knb1* had very rumpled leaves even at the level revealed by the scanning electron microscope. Therefore, it is still unclear whether more epicuticular wax was produced or whether computer-aided leaf-area determinations were an underestimate of actual surface area leading to higher total wax load estimates. In contrast to leaves, the stems of both *knb1* and *bcf1* had a degree of glaucousness similar to that of wild-type stems. However, wild-type, *knb1*, and *bcf1* stem epicuticular wax crystallization patterns were slightly different from one another. At present it is un-

clear whether the small changes in *knb1* and *bcf1* wax chemistry or other factors in the secretory pathway caused these differences.

In contrast to the increased glaucousness of *knb* and *bcf* leaves, *wax* leaves were more glossy than wild-type leaves. The dramatic reductions in overall leaf epicuticular wax load or changes in other characteristics associated with the leaf-fusion phenotype of *wax* mutants may have contributed to their glossy leaf appearance. The *wax* mutant stems had increased glossiness, which was likely associated with decreased epicuticular wax crystal density.

Alterations in leaf epidermal cell shape of the *knb1* and *bcf1* mutants and the leaf epidermal cell size of *wax1* suggest that epicuticular wax secretory pathways through the cell walls and/or cuticle layers may have been altered. Jeffree et al. (1976) proposed that plant epicuticular wax precursors may simply diffuse to the surface through the cell walls and primary cuticle in submicroscopic spaces. However, Pyee et al. (1994) identified lipid-transfer proteins in the epicuticular wax of broccoli, raising the possibility that extra-cytoplasmic wax secretion is more complicated than simple diffusion. Theoretically, modifications in the microfibrillar organization or other properties of the cell wall could influence these intricate wax-secretory mechanisms, leading to altered epicuticular wax deposition on the surface. Evidence that alterations in leaf shape can be caused by alterations in cell-wall components has recently been presented (Reiter et al., 1993). Potentially, cell-wall mutants might have relaxed or loosened cell-wall structure that creates pores or channels for epicuticular wax precursor secretion. The flake-like wax crystals on leaves of *knb* and *bcf* mutants having altered epidermal cell shape might indicate accumulation of epicuticular waxes above newly created pores in altered cell walls. Studies are in progress to determine whether *knb*, *bcf*, or *wax* mutants have modifications in their cell walls or cuticles that might influence epicuticular wax secretion.

The *bcf1* mutants were 1-alcohol deficient in their leaf epicuticular waxes similar to *cer4* in Arabidopsis (Jenks et al., 1995). Previously, McNevin et al. (1993) and Jenks et al. (1995) hypothesized that the *cer4* 1-alcohol deficiency was due to inhibited activity of an aldehyde reductase. However, the dramatic alterations in leaf morphology of *bcf1* leads us to reject this same simplistic explanation for the *bcf1* leaf 1-alcohol deficiency. Modifications within the *bcf1* cell walls, cuticles, or other locations within the secretory pathway may have caused selective suppression of 1-alcohol secretion to the surface.

Epidermal cells from various organs of *wax1* fused with others of the same plant during early organ development as reported for the Arabidopsis mutants *fdh1* (Lolle et al., 1992), *cer10*, and *cer13* (M. Jenks, unpublished data). However, considerable evidence suggests that *wax1* differs significantly from both the *fdh* and *cer* mutants. Of more than 100 independent *cer* mutants previously described (Koornneef et al., 1989; McNevin et al., 1993), none were found to possess glossy leaves. In addition, *wax1* mutants do not form the fiddlehead-shaped floral structures like *fdh1*, *cer10*, and *cer13*. Leaves and other organs of the *wax1* and

fdh1 mutants often fuse; however, only floral fusions have been recorded for *cer10* and *cer13* under our conditions. Moreover, *fdh1* and the eight other independently occurring *fdh* mutants have normal stem glaucousness (S. Lolle, personal communication), and *cer10* and *cer13* have only slightly reduced stem glaucousness. By comparison, *wax1* has very glossy stems. The *fdh1* stems have normal epicuticular wax chemical profiles (M.A. Jenks, unpublished results), whereas *cer10* and *cer13* stems have epicuticular waxes with increased chain-length distributions for alkane and 1-alcohol constituents. By comparison, *wax1* stems have a dramatic reduction in overall epicuticular wax load compared to *cer10* and *cer13*, alkane and 1-alcohol chain lengths that are comparable to those of wild type, and a unique increase in 2-alcohol/alkane and ketone/alkane precursor ratios. Similarly, leaves of *cer10* and *cer13* mutants have a much smaller reduction in overall leaf wax load than *wax1* and a much different chain-length distribution for major epicuticular wax constituents. The *fdh1*, *cer10*, and *cer13* mutants are fertile and have a determinant growth habit, whereas *wax1* is both male and female sterile and has a comparatively indeterminate growth habit and more thin and twisted stems.

Lolle et al. (1992) hypothesized that *fdh1* organ fusion may be due to mutation-induced diffusion of a chemical signal that causes epidermal cells to fuse. Future studies may show that the complex phenotypes of *wax1*, *fdh1*, *cer10*, and *cer13* are due to altered secretion of epicuticular wax precursors and morphogenic factors that are unidentified at present.

Previous studies identified the first plant cuticle proper mutants among a *Sorghum bicolor* epicuticular wax mutant collection (Jenks et al., 1994a). These cuticle mutants had the lowest total epicuticular wax load and wilted much faster than all other *Sorghum* epicuticular wax mutants (Jenks et al., 1992, 1994a). Similarly, the Arabidopsis *wax1* mutant had lower total epicuticular wax on leaves and wilted more rapidly on the laboratory bench than any of the 21 *cer* mutants (Jenks et al., 1995; M. Jenks, unpublished data). Studies are currently underway to determine whether the *wax* mutants have an altered cuticle proper. Moreover, epidermal surfaces that contact during early organ development fuse together in *wax* mutants but not in wild type. Future studies may show that the cuticle and epicuticular wax layers on *wax* mutants have been so reduced that they no longer suppress transmission of factors that allow developing organs to fuse. Conversely, *wax1* could have reduced secretion of both surface lipids and a morphogenic factor that suppresses epidermal fusion. Further studies are needed.

The 2-alcohols and ketones in plant epicuticular waxes are thought to arise from enzymatic oxidation reactions starting with alkanes (Kolattukudy et al., 1973). Cheesbrough and Kolattukudy (1984) presented evidence that aldehyde decarbonylation to alkanes may occur in the plant cell wall or cuticle layers. Potentially, the oxidative reactions that convert alkanes to 2-alcohols and ketones might also occur in the cell wall or cuticle layers. Our results showing relatively high C_{29} 2-alcohol/C_{29} alkane

and C_{29} ketone/C_{29} alkane ratios on *wax1* stems compared to wild type could be explained by either an increase or decrease in the secretory efficiency of epicuticular wax precursors. For example, altered secretion could have increased conversion of alkanes to 2-alcohols and ketones before they are released to the surface or increased wax transport back into epidermal cells and thereby increased conversion of alkanes to 2-alcohols and ketones. Previous studies have shown that epicuticular wax constituents can reenter the epidermal cell cytoplasm (Cassagne and Lessire, 1975). Another explanation for the *wax1* alteration in these ratios is that the alkane hydroxylase and 2-alcohol oxidase efficiencies have perhaps increased to more rapidly utilize the alkane substrate pool. Studies are in progress to determine whether *wax1* has altered secretory mechanisms or other factors controlling the relationship between alkanes and their oxidized products.

Lipid and cell-wall constituents are two major secretory products of plant epidermal cells. However, little is known about how these secretory products are transported from their cytoplasmic sites of synthesis through the plasmalemma (Moore and Staehelin, 1988; Jenks et al., 1994b). For instance, does the secretion of lipid and cell-wall constituents use separate or identical secretory pathways? Could the *knb*, *bcf*, and *wax* mutant phenotypes be caused by altered secretion of cell-wall constituents or formation of the cell-wall structure itself? Since plant cell shape is determined by cell-wall morphology, mutants with altered cell shape likely possess alterations in the precise structure of their cell walls. The *knb1*, *bcf1*, and *wax1*, mutants having both altered cell shape and altered epicuticular wax deposition, may be useful in future studies coupling EM and substrate and antibody labeling to shed light on cell-wall pathways used in epicuticular wax precursor secretion.

The results of our simple visual screen for leaf glaucousness in a T-DNA-mutagenized Arabidopsis population uncovered eight new mutants with defects in leaf morphology and epicuticular wax profiles, which were divided into three distinct classes based on overall plant morphology. These mutants should prove valuable in future studies to elucidate both the complex secretory processes in epicuticular wax production and interesting developmental phenomena.

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